Peripheral tissue release of interleukin-6 in patients with chronic kidney diseases: Effects of end-stage renal disease and microinflammatory state

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To examine if uremia influences muscle interleukin-6 (IL-6) metabolism we studied the exchange of IL-6 across the forearm in 16 patients with chronic kidney disease (CKD) (stages 3 and 4), in 15 hemodialysis (HD)-treated end-stage renal disease (ESRD) patients ($n = 15$), and in six healthy controls. In addition, we performed an analysis of both IL-6 protein and IL-6 mRNA expression in muscle of CKD (stage 4) patients showing evidence of inflammation and in controls. A release of IL-6 from the forearm was observed in patients with elevated IL-6 plasma levels. Arterial IL-6 was directly related to released IL-6 ($r = 0.69$; $P < 0.004$) in HD patients. Both IL-6 protein and IL-6 mRNA expression were increased in muscle of inflamed CKD patients vs controls ($P < 0.05$). Although muscle net protein balance was similar in all patients, it was significantly more negative in HD patients with high than in those with low IL-6 plasma levels ($P < 0.05$). In addition, net protein balance was related to the forearm release of IL-6 in HD patients only ($r = 0.47$; $P < 0.038$). These data demonstrate that IL-6 expression is upregulated in muscle, and that muscle tissue, by releasing this cytokine, may contribute to the inflammatory response in HD patients. The release of IL-6 from peripheral tissues is associated with an increase in muscle protein loss in HD patients, suggesting that muscle release of IL-6 is linked to protein catabolism in these patients. The release of IL-6 from peripheral tissues may act as a signal for the inflammatory response and contribute to functional dysregulation in uremia.

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KEYWORDS: interleukin-6; inflammation; skeletal muscle; uremia

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Several studies have shown a strong association between chronic inflammation and long-term mortality and morbidity in patients with end-stage renal disease (ESRD).[1,2] The percentage of patients showing evidence of inflammation increases progressively along with the decline in renal function, suggesting that cell release and/or body removal of pro-inflammatory cytokines is altered by uremia.[3] Interleukin-6 (IL-6) is a major factor coordinating the acute-phase response[4] and plasma levels of IL-6 are associated with increased cardiovascular risk[5] and loss of muscle mass,[6] both in the general population and in ESRD patients.[7] However, sites and mechanisms responsible for the regulation of circulating IL-6 and other cytokines in humans are currently poorly known. Although most of the circulating IL-6 is secreted from activated macrophages and lymphocytes, adipocytes[8] and skeletal muscle[9] are also a possible source of this cytokine. IL-6 mRNA is expressed in resting human muscle and is rapidly increased by contraction.[9] A release of IL-6 from the legs (which are mainly composed of skeletal muscle) has been shown to take place during physical exercise or glycogen depletion.[9,10] In addition, it has been recently observed that insulin increases IL-6 gene expression in insulin-resistant, but not in healthy skeletal muscle[11] and that IL-6 is released by the forearm muscle in obese subjects.[12] Both reactive oxygen species[9,10] and lipopolysaccharide[9,10] can upregulate muscle IL-6, likely because of an activation of nuclear factor, nuclear factor-κB. Muscle-derived IL-6 could play a physiological role to maintain metabolic homeostasis by stimulating lipolysis during periods of increased metabolic demand. In addition, in the context of skeletal muscle, IL-6 has variously been reported to regulate carbohydrate metabolism, increase satellite cell proliferation, or cause muscle wasting.[11–14] Therefore, available data indicate that several physiologic and pathological stimuli are able to prime the mechanisms for muscle IL-6 release.

Whether uremia induces changes in muscle IL-6 metabolism has never been examined. The aim of the present study was to explore the hypothesis that IL-6 could be locally produced in skeletal muscle and exported to other tissues.

First, we studied the exchange of this cytokine across the forearm (which is mainly made of skeletal muscle) in patients with chronic kidney disease (CKD) (stages 3 and 4) and in hemodialysis (HD) patients. In addition, in order to further explore whether muscle cells *per se* are the source of the elevation in IL-6, we performed an analysis of both IL-6 protein (by immunohistochemistry) and IL-6 mRNA expressions in muscles of patients with severe chronic renal failure showing evidence of inflammation.

RESULTS

IL-6 balance across peripheral tissues

Plasma flow across the forearm was 1.9 ± 0.13, 2.2 ± 0.15, and 2.2 ± 0.30 ml/min/100 ml, respectively, in CKD, ESRD patients, and control subjects ($P =$ NS).

Individual arterial and venous levels of IL-6, as well as their exchange rates across the forearm are reported in Table 1. Arterial levels of IL-6 in controls were within the range of those previously reported in arterial plasma.[15] In addition, IL-6 levels in the deep forearm vein, although tendentiously higher, were not statistically different from their corresponding arterial level. Similar findings have been obtained across the forearm[8] and the leg[12] in healthy subjects.

Arterial IL-6 levels were >5 pg/ml in seven CKD and 10 ESRD patients. In these patients, the deep venous IL-6 levels were significantly higher than the corresponding value in the artery (Table 2). A release from peripheral tissues was observed only as a trend in CKD patients who showed IL-6 levels <5 pg/ml. When considered as a whole group, the negative arterio-venous difference for IL-6 was statistically significant ($P<0.008$) in CKD and ESRD patients. The release of IL-6 from peripheral tissues was about sevenfold increased in HD patients with high IL-6 vs the corresponding value in CKD patients. Arterial IL-6 was directly related to peripheral release of IL-6 in HD patients ($r = 0.69$; $P<0.004$) (Figure 1a), suggesting that release from periphery can influence plasma IL-6 levels. However, this association was not statistically significant in CKD patients ($r = 0.331$; $P =$ NS) (Figure 1b).

Cytokine arterial plasma levels were greater in patients with CKD than in controls (IL-1$\beta = 6.5 \pm 1.9$, IL-10 $= 2.7 \pm 0.2$, and tumor necrosis factor-$\alpha = 33.0 \pm 3.2$ pg/ml in patients vs IL-1$\beta = 3.0 \pm 1$, IL-10 $= 1.7 \pm 0.2$, and tumor necrosis factor-$\alpha = 11.5 \pm 2$ pg/ml in controls, $P<0.05$–0.01). Plasma levels of these cytokines tended to be further increased in HD patients (IL-1$\beta = 9.3 \pm 2.0$, IL-10 $= 5.8 \pm 0.3$, and tumor necrosis factor-$\alpha = 93 \pm 12$ pg/ml, $P<0.05$-0.01 vs controls). However, no significant arterio-venous gradient across the forearm was observed for these cytokines both in control subjects and each patient category (data not reported).

The evaluation of cardiovascular profile yielded a cardiovascular score higher in inflamed vs non-inflamed HD patients (3.78 ± 0.32 vs 2 ± 0.6, $P<0.02$). Conversely, this score was similar in inflamed vs non-inflamed CKD subjects (2.0 ± 0.32 vs 2.0 ± 0.29). The release of IL-6 from the forearm was linearly, directly related to the cardiovascular

Table 2 | Characteristics of the patients

	Forearm balance study (CKD patients)	Forearm balance study (HD patients)	Muscle biopsy study (CKD patients)
Gender (M/F)	13M/3F	12M/3F	7M/8F
Age (years)	66 ± 2	67 ± 3	69 ± 3
Body weight (kg)	73 ± 4	68 ± 4	67 ± 4
Height (cm)	169 ± 3	168 ± 3	163 ± 2
BMI (kg/m^2)	26 ± 1	24 ± 1	25 ± 1
Fat-free mass (kg)	49 ± 2	46 ± 8	45 ± 2
Fat mass (kg)	25 ± 2	21 ± 2	22 ± 3
nPNA (g/kg)	0.90 ± 0.1	1 ± 0.1	0.85 ± 0.1
Estimated GFR (ml/min.1.73 m^2)	24 ± 2	$2 \pm 1^{b,c}$	8.4 ± 1^{b}
Serum creatinine (mg/dl)	3.0 ± 0.2	$10 \pm 1^{b,c}$	6.8 ± 0.4^{b}
Serum albumin (g/dl)	3.5 ± 0.03	3.4 ± 0.13	3.5 ± 0.2
BUN (mg/dl)	61 ± 5	84 ± 8^{a}	84 ± 3^{a}
Bicarbonate (mmol/l)	23.1 ± 0.50	22.0 ± 0.90	23.2 ± 0.9
CRP (mg/l)	12 ± 3	35 ± 8^{a}	28 ± 8
Hemoglobin (g/dl)	12 ± 1	10.5 ± 1	11.3 ± 0.3
Cardiovascular score[d]	2.08 ± 0.23	2.94 ± 0.35	2.13 ± 0.46

BMI, body mass index; BUN, blood urea nitrogen; CKD, chronic kidney disease; CRP, C-reactive protein; F, Female; HD, hemodialysis; M, male; nPNA, normalized protein nitrogen appearance; GFR, glomerular filtration rate. Significance of difference vs the forearm balance study: $^{a}P<0.05$; $^{b}P<0.001$. Data are expressed as mean $\pm$ s.e.m. Significance of difference vs the muscle biopsy study: $^{c}P<0.05$. Cardiovascular score was obtained by the use of a standardized four-level scale based on atherosclerotic events.[25]

Table 1 | Forearm exchange of interleukin-6 in patients in controls

	Artery (pg/ml)	Forearm vein (pg/ml)	A−V difference	Rate (pg/min 100 ml)
Controls (n=6)	3.2 ± 0.98	3.5 ± 0.99	-0.2 ± 0.20	-0.5 ± 0.4
CKD (IL6 >5 pg/ml, n=7)	$28.8 \pm 6.2^{d,e,f}$	29.6 ± 6.26^{a}	-0.8 ± 0.33	-1.5 ± 0.49
CKD (IL6 <5 pg/ml, n=9)	2.5 ± 0.48	2.8 ± 0.54	-0.3 ± 0.25	-0.9 ± 0.45
CKD (all subjects, n=16)	14.0 ± 4.26	14.5 ± 4.31^{a}	-0.5 ± 0.17	-1.12 ± 0.28
ESRD (IL6 >5 pg/ml, n=10)	$44.1 \pm 7.36^{d,e}$	49.6 ± 8.54^{b}	-5.5 ± 1.82	$-11.1 \pm 3.40^{d,h}$
ESRD (IL6 <5 pg/ml, n=5)	3.30 ± 0.58	3.34 ± 0.56	0 ± 0.03	0 ± 0.75
ESRD (all subjects, n=15)	30.5 ± 7.05^{d}	34.4 ± 8.08^{c}	-3.7 ± 1.38	-7.4 ± 2.66^{g}

Data are expressed as mean $\pm$ s.e.m. Significance of difference of arterial (A) vs venous (V) concentration: $^{a}P<0.05$, $^{b}P<0.035$, $^{c}P<0.02$.
Significance of difference vs controls: $^{d}P<0.05$ or less. Significance of difference vs the corresponding value in patients with low IL-6: $^{e}P<0.01$. Significance of difference vs the corresponding value in ESRD patients with low IL-6: $^{f}P<0.001$. Significance of difference vs the corresponding value in CKD (all subjects): $^{g}P<0.05$. Significance of difference vs the corresponding value in CKD patients with high IL-6: $^{h}P<0.05$.CKD, chronic kidney disease; ESRD, end-stage renal disease; IL-6, interleukin-6.

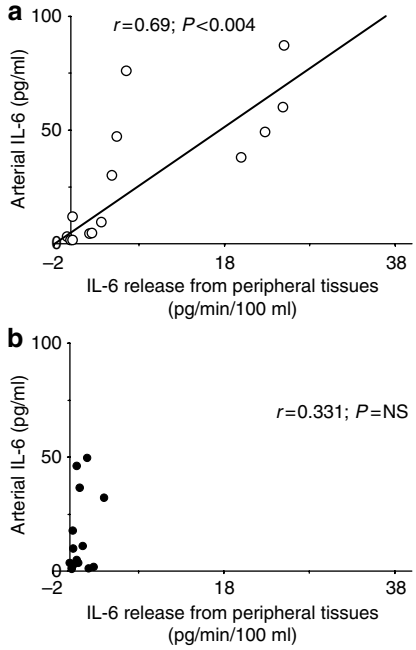

Figure 1 | Relationship between arterial IL-6 and its release from peripheral tissues (a) in HD-treated patients with ESRD or (b) with CKD (stages 3 and 4). Arterial IL-6 levels were related to the release of IL-6 from peripheral tissues in HD patients, but not in CKD patients.

score in HD ($r=0.603$; $P<0.003$), but not in CKD patients ($r=0.08$; $P=$NS).

C-reactive protein (CRP) levels correlated directly with forearm release of IL-6 ($r=0.72$; $P<0.001$). However, when patients were considered separately, this association persisted to be statistically significant in HD patients only (HD patients $r=0.70$, $P<0.002$; CKD patients, $r=0.24$, $P=$NS). No relationship was observed between indexes of obesity (body mass index and body fat mass), plasma bicarbonate, hemoglobin, uric acid, residual renal function, and the release of IL-6 from the forearm.

Net protein balance across the forearm

Both in patients and controls, the deep venous phenylalanine levels exceeded the arterial ones ($P<0.01$), thus indicating phenylalanine release from the forearm and proteolysis. Net phenylalanine balance across the forearm in CKD and in dialyzed ESRD patients was similar to controls (Figure 2). When considering CKD patients separately according to IL-6 levels, net protein balance was also not different in patients with high vs low plasma IL-6. However, net phenylalanine balance was significantly more negative (indicating a decrease in muscle protein synthesis or an increase in protein degradation) in HD patients showing evidence of microinflammation as compared to non-inflamed HD subjects. Net phenylalanine balance was weakly related to IL-6 release from the forearm when considering together CKD and ESRD patients ($r=0.365$; $P<0.1$). However, a significant relation between forearm phenylalanine and IL-6 release was again

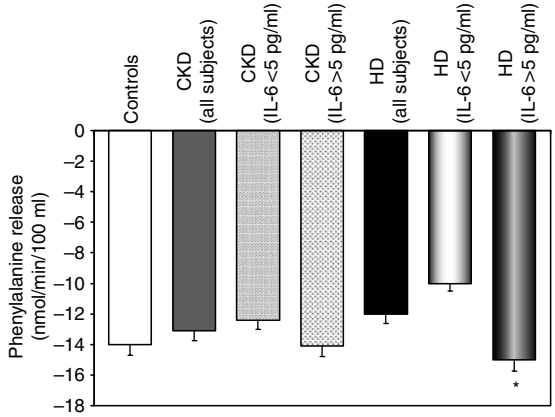

Figure 2 | Net protein balance across the forearm in the post-absorptive, basal state in patients with CKD (stages 3 and 4) and in HD-treated patients with ESRD. Data are expressed as mean ± s.e.m. *$P<0.05$ vs HD patients without inflammatory response.

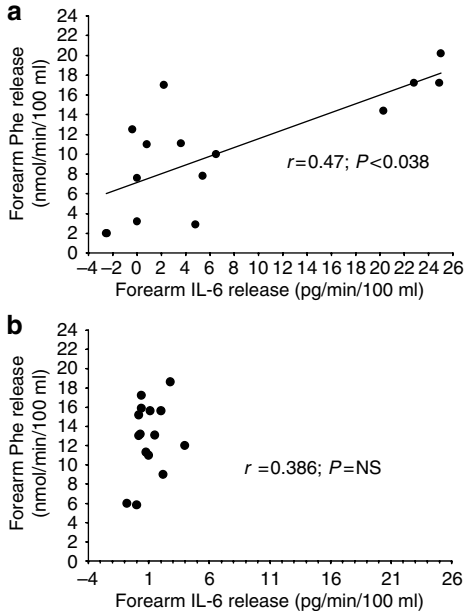

Figure 3 | Relationship between the release of phenylalanine and that of IL-6 from peripheral tissues (a) in HD-treated patients with ESRD and (b) in patients with CKD (stages 3 and 4). The release of phenylalanine by peripheral tissues increased progressively along with the release of IL-6. This correlation was observed in HD, but not in CKD patients.

observed in HD but not in CKD patients ($r=0.469$, $P<0.037$) (Figure 3a and b).

Muscle biopsy studies

Patients studied in this group displayed evidence of an inflammatory response (plasma CRP 28 ± 5 mg/dl; IL-6 15 ± 2 pg/ml). As Figure 4 shows, we were able to detect IL-6 mRNA in muscles, both in patients and in controls. Muscle IL-6 mRNA was, however, 2.4-fold increased ($P<0.05$) in patients vs controls.

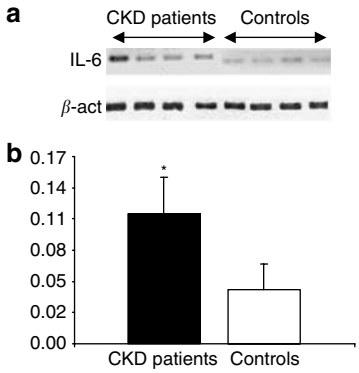

Figure 4 | IL-6 mRNA expression is increased in uremic muscle. Total RNA was extracted from *Rectus Abdominis* muscles of control and CKD patients. Semiquantitative PCR was performed for IL-6 and β-actin (see Materials and methods). Representative reverse transcriptase-PCR analysis of skeletal muscle IL-6 and β-actin messages in four consecutive patients and controls (**a**). Densitometric values for IL-6 mRNA were normalized for β-actin expression, and the ratio of IL-6 to β-actin was determined (**b**). Values are means $\pm$ s.e.m. *Significantly different ($P<0.05$) vs controls

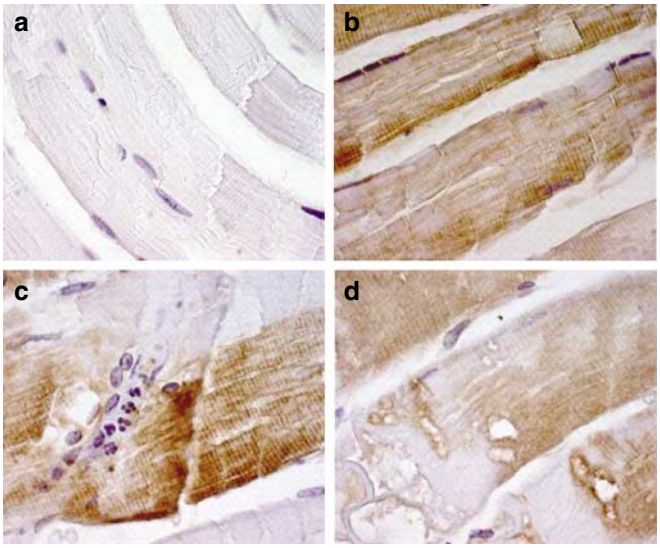

Figure 5 | IL-6 expression (immunostaining) is increased in uremic muscle. Immunohistochemical analysis was performed with an anti-human IL-6 polyclonal antibody in *Rectus Abdominis* muscles of control and CKD patients. IL-6 protein was visualized by image analysis. Control subject (**a**) and patients with CKD and microinflammation (**b**, **c**, **d**). (original magnification $\times$ 1000).

The IL-6 staining was absent in muscle tissue from healthy subjects. Representative images are shown (Figure 5a and b) for one control subject and three CKD patients. In CKD patients, the expression of IL-6 was clearly detectable (Figures 5 and 6). The IL-6 staining appeared as a diffuse staining of the cytoplasm of skeletal muscle fibers in all subjects. There was no IL-6 staining present between muscle fibers. In addition, mononuclear infiltrates were negative for IL-6 immunostaining.

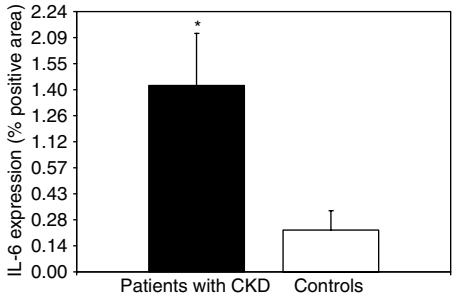

Figure 6 | Results of IL-6 image analysis (immunostaining) in patients with CKD ($n=10$) and controls ($n=6$) in the resting state. Values are means $\pm$ s.e.m. *Significantly different ($P<0.05$) vs controls.

DISCUSSION

In the present study, we tested the hypothesis that IL-6, a major mediator of the acute-phase response, is released by skeletal muscle both in patients with moderate advanced CKD and those with ESRD displaying evidence of an inflammatory response. This issue has been assessed by multiple determinations, including the measure of IL-6 balance across the forearm (which is mainly made of skeletal muscle), immunohistochemical evaluation of IL-6 in muscle, and muscle detection of IL-6 mRNA. First, we observed that IL-6 is released by peripheral tissues into the systemic circulation in patients with evidence of inflammation. On the contrary, no significant gradient of IL-6 occurs across peripheral tissues both in healthy controls and patients without evidence of an inflammatory response. In addition, we observed that IL-6 gene and protein expression are upregulated in skeletal muscle of patients with advanced CKD displaying an inflammatory response. Accordingly, the peripheral output of IL-6 in uremia can be attributed to an increase in IL-6 gene transcription within skeletal muscle and translation of IL-6 protein that is subsequently released.

Our IL-6 tissue immunohistochemical findings in uremic patients are similar to those reported by Febbraio *et al.*,[9] who showed that IL-6 protein was distributed homogenously across muscle fibers in exercising healthy subjects. In our study, although only scanty mononuclear infiltrates were observed across muscle fibers in CKD patients, these were negative for IL-6 immunostaining (Figure 5). These considerations suggest that IL-6 is directly produced by muscle cells.

Recently, it has been estimated that 10–35% of the body's basal circulating IL-6 is derived from adipose tissue.[16] According to our data, the output of IL-6 from peripheral tissues can play a major role in influencing circulating levels of this cytokine in patients with CKD. Results obtained in healthy subjects that no significant enrichment or depletion of IL-6 occurs across the forearm muscle are in accordance with previous findings obtained across the forearm[8] and leg[17] in the normal condition. The released IL-6 output from the forearm observed in CKD patients is of the same magnitude observed recently in obese, insulin-resistan subjects.[12]

According to the measure of the forearm arterio-venous gradient of IL-6 in obese subjects, the estimated contribution of IL-6 release from skeletal muscle to systemic IL-6 is about 12%, varying from 2 to 42%.[12] In our study, we observed that the IL-6 output was fairly matched by variations in arterial levels of the same cytokine in HD, but not in CKD patients. The correlation coefficient of the relation is 0.69, indicating that about 48% of variations in arterial IL-6 were explained by variations in IL-6 release from periphery in HD subjects. According to the measurement errors in each variable, this indicates a strong association, and suggests that release by muscle plays a major role in determining IL-6 plasma levels in HD patients. The reason(s) why such an association was not found in patients with less-advanced renal disease may include on one hand, the residual metabolic activity of the kidney, which can remove IL-6 from blood (unpublished data from our laboratory), and, on the other hand, a more marked inflammatory condition in hemodialysis-treated patients.

Which signals might trigger IL-6 release from muscle in uremia? Several upstream factors have been shown to be able to induce the transcription of this cytokine in human muscle *in vitro* or *in vivo*. IL-6 synthesis is activated by intracellular calcium levels, mitogen-activated protein kinases, and other cytokines such as IL-1β.[9] Nutritional factors, such as low glycogen availability, can also increase IL-6 transcription.[17] Of note, the uptake of glucose by skeletal muscle is blunted[18] and muscle glycogen depletion have been reported by some studies in uremic patients. In addition, reactive oxygen species can upregulate muscle IL-6, likely because of an activation of nuclear factor, nuclear factor-κB.[9,10,19]

Another possible signal is related to metabolic acidosis. Metabolic acidosis contributes to the regulation of synthesis of inflammatory cytokines in circulating cells, and, possibly, in skeletal muscle.[20,21] In this regard, we were not able to find a relation between bicarbonate levels and forearm release of IL-6. However, most of the patients in our study displayed bicarbonate levels in the normal range.

We conclude that IL-6 expression is upregulated in muscle, and that forearm muscle releases substantial amounts of IL-6, a major mediator of the acute-phase response, in patients with CKD or ESRD and with evidence of inflammation. The relation between arterial IL-6 and CRP levels suggests that release of this cytokine from periphery may act as a signal for the inflammatory response. However, the biological effects of muscle-derived IL-6 in physiological and pathologic states are not completely ascertained. IL-6 released from muscle might behave as a hormone to increase substrate delivery.[8] One of the effects of IL-6 is to stimulate lipolysis, causing the release of NEFA from the adipocyte. This effect may be beneficial to sustain body needs during exercise, but could be harmful in chronic illness conditions.[22] In addition, IL-6 might behave on skeletal muscle as an autocrine factor. Elevated levels of IL-6 are associated with a reduction in muscle mass and muscle strength[23] When administered to animals, IL-6 can cause muscle atrophy with changes in the balance of growth-factor-related signaling favoring a catabolic profile.[13] If so, peripheral tissues could play a double role of victim and culprit of the inflammatory response in uremia. In our study, net protein balance across peripheral tissues was greater in inflamed vs non-inflamed HD patients. In addition, the release of IL-6 correlated with the negative protein balance in HD but not in CKD patients. This indicates that inflammation may affect the control of protein balance in HD patients, by acting either directly or indirectly, on protein synthesis and/or degradation. It is of note that such a relation was not observed in CKD patients, even in those displaying an inflammatory response. It is unclear if this was due to a lower degree of inflammation in this patients subgroup or to catabolic mechanisms specifically activated in HD-treated patients. With this regard, it is interesting that it has been recently observed that hemodialysis acutely increases IL-6 gene expression in skeletal muscle.[24] Taken together, our data show that the effects of muscle-derived IL-6 on protein metabolism, as well as its endocrine/autocrine effects should be fully explored in renal patients as well as in other chronic conditions. Understanding these processes should allow for a new therapeutic approach to uremia and its complications.

MATERIALS AND METHODS

Forearm exchange of cytokines

Thirty-one patients with CKD participated in this study (Table 2). Patients had no evidence of gastrointestinal or liver disease, malignancy, or intercurrent infection. Sixteen patients had moderate to severe CKD with estimated[25] glomerular filtration rate of 24 ± 2 ml/min (range 13–38); their systolic and diastolic arterial pressures were 150 ± 11 and 88 ± 9 mm Hg, respectively. Fifteen were maintenance HD patients (mean dialytic age 27 months, range 6–72; K_t/V 1.35 ± 0.1). Their systolic and diastolic arterial pressure were 155 ± 12 and 90 ± 7 mm Hg, respectively. Causes of renal diseases were glomerulonephritis (10 patients), hypertensive nephrosclerosis (15 patients), tubulointerstitial nephritis (three patients), type II diabetic nephropathy (two patients), and polycystic renal disease (one patient). Patients were taking drugs, including antihypertensive drugs, sodium bicarbonate, calcium carbonate, and erythropoietin, which were prescribed as appropriate for each individual. An increase in CRP levels (>10 mg/l) and signs of cardiovascular disease (coronary artery disease or peripheral artery disease) were present in seven patients with CKD and 10 patients with ESRD. Cardiovascular profiles in each patient were evaluated by a standardized scale based on atherosclerotic events (coronary heart disease, cerebrovascular disease, and peripheral vascular disease).[25] Three of the CKD and six of the ESRD patients were moderately malnourished according to subjective global nutrition assessment. Estimated calorie intake was 30–32 and 28–32 kcal/kg, respectively, in CKD and ESRD patients, respectively.

Control subjects (4M/2F, age 61 ± 6 years, body mass index 24 ± 4 g/m^2) were on a diet providing 31–35 kcal and 1–1.2 g of protein/kg/day. Routine laboratory tests, acid–base and electrolyte measurements were all normal.

The study was part of a larger protocol on protein metabolism in CKD approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa. All subjects were informed about the nature, purposes, procedures, and possible risks of the study before their informed consent was

obtained. The procedures were in accordance with the Helsinki declaration.

Protocol

Patients were studied in the post-absorptive state, at rest. To avoid possible interferences of the dialytic procedure, HD patients were studied after approximately 72–74 h from the last dialytic treatment. Catheters were placed percutaneously into a brachial artery and an ipsilateral retrograde cubital vein. After a 30-min period, triplicate sets of arterial and venous samples were taken at 20-min intervals during a 60-min period. The oxygen saturation in the forearm venous samples was less than 60%, implying that deep venous blood was actually sampled. Forearm blood flow was measured by strain-gauge plethysmography.[26,27] Flow was expressed per 100 ml forearm volume.

Blood was collected in (ethylenediaminetetraacetic acid) tubes, which were immediately centrifuged at 6000 r.p.m. for 10 min at $+4^{\circ}$C. Plasma was quickly separated from blood cells and stored at -80°C until assayed. Plasma IL-6 concentrations were determined by a high sensitivity human IL-6 enzyme-linked immunosorbent assay technique (Diaclone, Besancon, France). IL-1, IL-10, and tumor necrosis factor-α were also measured in plasma using enzyme-linked immunosorbent assay kits (Diaclone, Besancon, France) according to the manufacturer's directions. Assays were performed in triplicate. The inter- and intra-assay coefficient of variance % for each cytokine were $<5\%$. In particular, the IL-6 assay had a limit of detection of 0.8 pg/m, intra-assay coefficient of variance of 2.8%, and interassay coefficient of variance of 1.4%. All samples from one individual were always run in the same batch. Samples in the high range (>50 pg/ml) were appropriately diluted.

Net protein balance across the forearm was measured from the release of phenylalanine.[27] Net protein balance represents the difference between protein synthesis and degradation. In the postabsorptive condition, as evaluated in the present study, the net balance of phenylalanine across the forearm is negative because protein degradation is greater than protein synthesis.

Hematocrit was measured by a microcapillary procedure and serum CRP by nephelometry.

Calculations

The exchange of IL-6 and phenylalanine across the forearm was calculated by the Fick's principle: $((Xa)–(Xv)) \times$ plasma flow, where X_a and X_v are the concentrations of the metabolites in arterial and venous plasma, respectively. A cutoff value of 5 pg/ml was employed to separate patients with high or normal IL-6 values.[15]

Body composition was estimated by anthropometry.[28] Plasma flow was calculated from blood flow and hematocrit.

Studies on muscle biopsies

Muscle biopsies were obtained from *Rectus Abdominis* muscle of 15 ESRD patients (seven males, eight females) during the placement of a peritoneal dialysis catheter. Samples were taken in 12 otherwise healthy subjects (7M/5F, age 66 ± 4 years) from the *rectus abdominis* muscle during elective surgery for abdominal wall hernias (controls). Both these procedures were performed under local anesthesia. Clinical characteristics of patients are shown in Table 2.

Causes of renal diseases were hypertensive nephrosclerosis (10 patients), chronic glomerulonephritis (two patients), tubulointerstitial nephritis (two patients), and obstructive uropathy (one patients). All patients presented an increase in CRP levels. Two patients had isolated monolateral and 10 bilateral peripheral arterial disease. Coronary artery disease was observed in four patients. Immediately following each biopsy, muscle tissue was separated into two sections: one of these was used for RNA analysis and the other mounted in TissueTek (Fronine, Riverstone, Australia), frozen in isopentane (BDH Laboratory Supplies, Poole, UK) over liquid nitrogen, and stored at -80°C until later analysis.

Histological preparation and immunohistochemical staining for human IL-6 protein

Paraffin sections (5 μm) of 2% paraformaldeyde-fixed muscle (10 ESRD patients and six control subjects) were deparaffined, hydrated, and pretreated with pepsin for 15 min at 37°C and 0.01% NP-40 for 30 min. Samples were treated with 3% H_2O_2 in methanol for 15 min. Sections were incubated in primary antibody (1:200 in phosphate-buffer saline) (anti-human IL-6, rabbit polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at room temperature. Subsequently, sections were incubated with the secondary antibody (biotynilated immunoglobulin G 1:100 in phosphate-buffer saline) for 30 min. The biotin–streptavidin–peroxidase method and light microscopy examination were performed as previously decribed.[29]

Measurement of IL-6 mRNA in muscle biopsies by reverse transcriptase-polymerase chain reaction

Total RNA was extracted by applying Trizol reagent (Invitrogen, Milan, Italy). Subsequently, the homogenized samples were incubated at room temperature for 15 min. Chloroform (0.2 ml/ml) was added to the solution and the total RNA was precipitated from the aqueous phase by adding isopropanol and incubated for 15 min at room temperature. This operation was followed by centrifugation for 15 min at 12 000 *g* at 4°C. After a rinse in 75% ETOH, the final pellets were re-suspended in 30/50 μl of nuclease-free water.

To check the quality of the RNA, sample aliquots were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The quantity of extracted RNA was measured spectrophotometrically at 260 nm. Complementary DNA (cDNA) was reverse transcribed (reverse transcriptase) from 0.5/1 μg of total RNA using 20 pmol/μl random hexamers and 5 U Improm II Reverse transcriptase (Promega Corp. Madison, WI, USA) in a final volume of 20 μl. To test the reverse transcriptase, the samples were amplified for a housekeeping gene, the β-actin. cDNA was amplified by polymerase chain reaction (PCR) reaction in a total volume of 25 μl of reaction mixture containing $1\times$ PCR buffer with $MgCl_2$ (Eppendorf, Hamburg, Germany), 0.2 mM each of dATP, dTTP, dCTP, dGTP, 0.25 mM of forward and reverse primers (gene of interest), and 0.5 U of *Taq* DNA polymerase (Eppendorf, Hamburg, Germany). Amplification products were subjected to electrophoresis through 1.5% agarose gels, purified with a DNA gel extraction kit (Qiaex-II,Qiagen, Milan, Italy), and used for sequence analysis. Genomic DNA and cDNAs were sequenced in an automatic sequencer.

PCR was performed in a Peltier Thermal Cycler dual block (PTC100 MJ, GMI, Ramsey, MN, USA) by use of the general cycle profile: 95°C for 5 min, as an initial denaturation, followed by 35 cycles of denaturation at 94°C for 45 s, specific annealing for gene of interest for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

The primers were designed from human IL-6 and β-actin mRNA sequence.[30,31] A 295-bp IL-6 gene fragment was amplified using the forward primer 5′-aaattcggtacatcctcgac-3′ and reverse primer

3′-caggaactggatcaggactt-5′(Invitrogen, Milan, Italy). A 219-bp β-actin gene fragment was amplified using the forward primer 5′-cccaaggccaaccgcgagaagat-3′ and reverse primer 3′-gtcccggccagc caggtccag-5′(Invitrogen, Milan, Italy). PCR products were separated by gel (1.5% agarose) electrophoresis, stained with ethidium bromide and visualized by ultraviolet illumination. Relative cytokine expression was determined in different samples, by comparing in-sample ratios (R) of the level of cytokine relative to the level of housekeeping gene.

Statistical analysis

The data regarding arterial and venous plasma IL-6, IL-6 mRNA, and β-actin were normally distributed. Statistical analysis was performed using analysis of variance for repeated measures to compare arterial with venous data. One-way analysis of variance followed by *post hoc* analysis was used to compare data from different groups. Linear regression and correlation were employed to evaluate the relationship between two variables. A P-value of <0.05 was considered statistically significant. Data are expressed as mean$\pm$s.e.m.

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